OPTIMAL CONDITIONS FOR PRIMER-DEPENDENT TRANSCRIPTION OF POLY(dT) BY RNA POLYMERASE

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SUMMARY

Poly(dT)-directed poly(A) synthesis by DNA-dependent RNA polymerase of Escherichia coli can be made dependent on the presence of oligo(Ap)$_4$A by raising the incubation temperature to 50°C or, less effectively, by addition of rifampicin. Both procedures could be useful in a transcription approach to DNA sequence analysis.

INTRODUCTION

We are interested in the use of RNA transcripts for the sequence analysis of DNA, using oligonucleotide primers to synchronize the transcription. The main problem in this approach is the unfortunate ability of RNA polymerases to start chains de novo. We have, therefore, started to look for conditions in which elongation of primers is strongly favoured over chain initiation, using a model system with poly(dT) as template and (Ap)$_4$A as primer. Two conditions have been described already in this and similar model systems which lead to suppression of chain initiation relative to chain elongation: low substrate concentrations and omission of sigma factor[1,2]. We report here two additional conditions that render poly(dT)-directed poly(A) synthesis completely dependent on (Ap)$_4$A.

MATERIALS AND METHODS

Poly(dT) (2.3 S), poly(dA) (3 S) and the dephosphorylated ribo-oligonucleotide (Ap)$_4$A were purchased from Miles Laboratories. Molar

extinction coefficients per nucleotide of 7.6×10^3, 8.9×10^3 and 11.8×10^3 were used for poly(dT), poly(dA) and the oligomer, respectively[3].

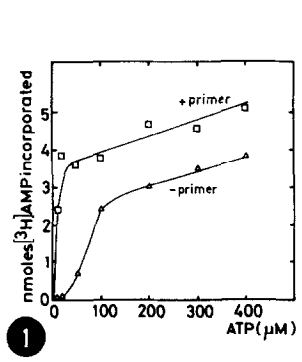
RNA polymerase from E.coli was purified by a modified Burgess[4] procedure, developed in Weissmann's laboratory (C.Weissmann, personal communication). The specific activity of the enzyme used was 170 units/mg protein. Unlabelled ribonucleotides were obtained from Sigma and the ^{3}H-labelled ribonucleotides were products of the Radiochemical Centre, Amersham.

The standard (0.2 ml) reaction mixture for the polymerization assay contained 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM K_2HPO_4, 0.1 mM dithiotreitol, 1 µg poly(dT) or poly(dA) and 10 µg enzyme. [^{3}H]NTP and ribo-oligomer $(Ap)_4A$ were added as indicated in the legends. The reaction was started by adding the enzyme, after preliminary incubation of all other reagents for 5-10 min at the indicated temperature, unless stated otherwise.

Incorporation of isotope was measured as acid-insoluble radioactivity, either by adding cold 5% trichloroacetic acid-60 mM sodium pyrophosphate after a 10-min incubation period, or in the case of the time-course experiments by transferring 20-µl aliquots from the reaction mixture to the trichloroacetic acid solution. After 100 µg of bovine serum albumin was added as carrier, the precipitates were collected on Whatman glass-paper discs, washed extensively, dried and counted in a Nuclear Chicago liquid scintillation counter.

RESULTS

Synthesis of poly(A) by RNA polymerase with poly(dT) as template shows a sigmoidal dependence on ATP concentration (Fig.1) and this sigmoidicity is abolished by the addition of the primer $(Ap)_4A$, which overcomes the rate-limiting initiation step. This con-



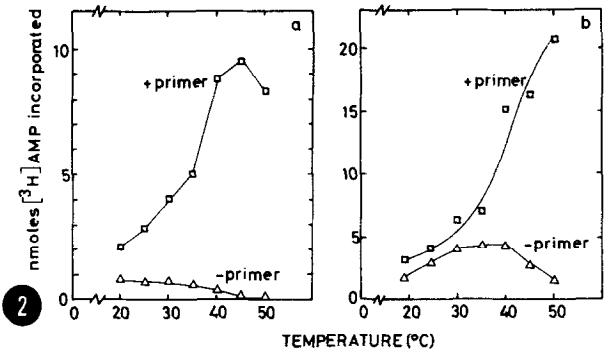
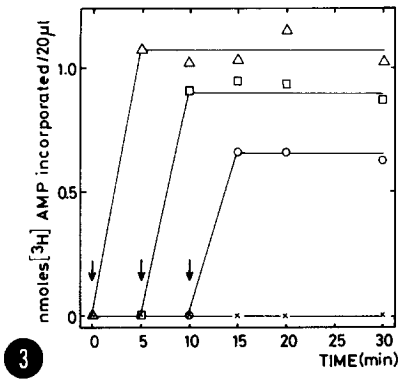


Fig.1. Effect of ATP concentration on poly(dT)-directed poly(A) synthesis with or without addition of the oligomer primer $(Ap)_4A$. Reaction conditions were as described in Materials and Methods. Incorporation was measured at 30°C after a 10-min incubation period, in the absence of added primer (Δ—Δ) and in the presence of 1.8 nmoles $(Ap)_4A$ (□—□).

Fig.2. Effect of temperature on poly(dT)-directed poly(A) synthesis. a) At low (50 μM) and b) at high (250 μM) ATP concentration, with or without addition of the oligomer primer $(Ap)_4A$. Reaction conditions were as described in Materials and Methods. Incorporation was measured after a 10-min incubation period at the indicated temperature in the absence of added primer (Δ—Δ) and in the presence of 1.8 nmoles $(Ap)_4A$ (□—□).

firms results of Niyogi[2] and similar results in other homopolymer-directed systems[1,5].

Fig.2 shows the effect of incubation temperature on poly(dT)-directed poly(A) synthesis at either high or low ATP concentration. Primer-dependent synthesis continues to increase with temperature up to 50°C with high ATP. With low ATP a plateau is reached at 40°C because ATP becomes limiting. Primer-independent synthesis on the other hand, rapidly declines at higher temperatures, especially at low ATP concentration. Hence, at 50°C and low ATP concentration, incorporation is completely dependent on the presence of primer. The primer effect is not merely due to the stabilization of the polymerase against heat inactivation. If the enzyme is preincubated with substrate and template, but without primer at 50°C, subsequent addition of primer gives a pronounced stimulation of poly(A) synthe-



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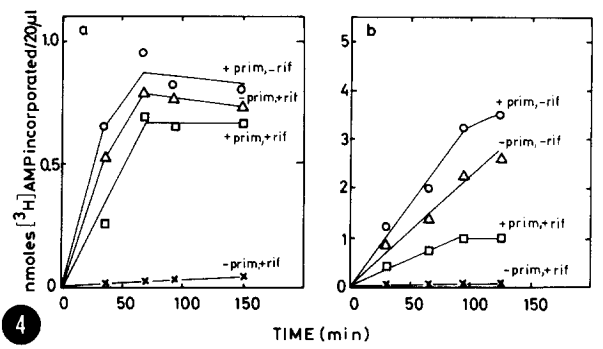

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Fig.3. Effect of primer $(Ap)_4A$ addition on poly(dT)-directed poly(A) synthesis at low ATP concentration (50 µM) and high temperature (50°C). Reaction conditions were as described in Materials and Methods. Incorporation was measured by taking 20-µl aliquots from the reaction mixtures at the times indicated; in the absence of added primer (X—X), and (arrows) with 1.8 nmoles $(Ap)_4A$ added at the onset of synthesis (Δ—Δ) and with 1.8 nmoles $(Ap)_4A$ added 5 min (□—□) or 10 min (0—0) after the reaction was started.

Fig.4. Effect of rifampicin (rif) on the $(Ap)_4A$ stimulation of poly(dT)-directed poly(A) synthesis at 30°C. a) At low (50 µM) and b) at high (250 µM) ATP concentration. Reaction conditions were as described in Materials and Methods, except that some reaction mixtures contained 1.8 nmoles of $(Ap)_4A$ primer and 9×10^{-6} M rifampicin. The solutions containing the template, buffer, $MgCl_2$, EDTA, dithiotreitol, K_2HPO_4 with or without primer, were heated 5 min at 65°C, cooled rapidly at 5°C and "annealed" for 45 min at 5°C. Then enzyme was added and the mixtures were preincubated for another 30 min at 5°C. After addition of rifampicin as indicated, all reactions were started by adding [^{3}H]ATP and transferring the tubes to 30°C. Incorporation was measured by taking 20-µl aliquots from the reaction mixtures at the times indicated: in the absence of primer and rifampicin (Δ—Δ), with primer and rifampicin (□—□), with primer minus rifampicin (0—0) and with rifampicin alone (X—X).

sis even after 10 min (Fig.3). Incorporation rapidly reaches a plateau in this experiment, because the ATP is limiting. We attribute the decrease in the incorporation plateau with increasing preincubation time to a slight ATPase activity in the enzyme preparation. It was not due to inactivation of enzyme or template because poly(A) synthesis immediately resumed when additional ATP was added at 25 min in the incubation in which primer was added after 10 min.

To be useful for sequence analysis, primer stimulation should

Table I: Specificity of $(Ap)_4A$-primed transcription at low (50 μM) ATP concentration

Reaction conditions were as described in Materials and Methods. The incorporation was measured after a 10-min incubation period at the temperatures indicated.

Template	Additions	nmoles NMP incorporated/10 min	
		30°C	50°C
None	ATP	<0.01	<0.01
	ATP + $(Ap)_4A$	<0.01	<0.01
Poly(dT)	ATP	0.7	<0.01
	ATP + $(Ap)_4A$	4	10
	UTP	not tested	<0.001
	UTP + $(Ap)_4A$	not tested	<0.001
Poly(dA)	UTP	0.15	<0.001
	UTP + $(Ap)_4A$	0.15	<0.001
	ATP	not tested	<0.01
	ATP + $(Ap)_4A$	not tested	<0.01

be specific. The experiments in Table I show that this is the case: there is no poly(A) synthesis in the absence of template, $(Ap)_4A$ does not stimulate UMP incorporation either on poly(dT) or on poly(dA) as template. Note that also with poly(dA) primer-independent synthesis is blocked completely at 50°C.

With the oligonucleotide-primed synthesis of poly(A) with poly(U) as template, maximal stimulation by the primer was found near the melting temperature of the poly(U)-oligomer complex[6,7]. This is not the case in our system, because the melting point of the poly(dT)-$(Ap)_4A$ complex is about 5°C (experiment not shown), i.e. at least 40°C below the temperature found for maximal poly(A) synthesis. We conclude that a pre-existent template-primer complex is not a prerequisite for primer stimulation. Apparently the enzyme is able to stabilize the template-primer complex to a remarkable degree.

As an alternative tool to restrict transcription to the elongation of oligonucleotide primers, Terao et al.[8] have used rifampicin, a drug known to inhibit specifically chain initiation, but not chain elongation by RNA polymerase[9]. They showed that addition of a heptanucleotide primer prevented the inhibition by rifampicin of the transcription of a single-stranded DNA and suppressed chain initiation in the absence of rifampicin. No protection against rifampicin was found, however, by Straat and Ts'o[10] when they studied the poly(U)-directed synthesis of poly(A) with $(Ap)_4A$ as primer. To see if this is due to a peculiarity of ribopolymer-directed RNA synthesis we repeated these experiments in our system. Fig.4 shows that $(Ap)_4A$ effectively protects against rifampicin at either high or low ATP concentration. Hence, poly(A) synthesis becomes primer dependent in the presence of rifampicin at 30°C, although dependence is less complete than at 50°C without rifampicin. We have also found a partial protection against rifampicin inhibition by $(Ap)_4A$ in poly(U)-directed poly(A) synthesis. Except for the nature of the polymerase, we cannot account for this discrepancy with the results of Straat and Ts'o.

The plateaus of incorporation in the presence of rifampicin and $(Ap)_4A$ primer were 2-3 times higher than the amount of poly(dT) input. We attribute this to the "slippage" phenomenon described by Chamberlin and Berg[11], since part of the product sedimented faster than the template poly(dT) in formaldehyde-sucrose gradients.

DISCUSSION

Initiation of RNA synthesis by RNA polymerase is a second-order reaction with respect to substrate concentration, whereas chain elongation is a first-order reaction. Hence, chain initiation becomes limiting at low substrate concentrations and this limitation

can be overcome by adding the appropriate primer oligonucleotides[12]. Our results show that RNA synthesis in the absence of primer is increasingly inhibited when the temperature is raised to 50°C and this must be an effect on chain initiation, since the rate of synthesis in the presence of primer is markedly stimulated. The actual target of this inhibiting effect is yet unknown. Although the sigma-factor is reported to be almost completely inactivated after 5 min at 50°C (ref.13), preliminary experiments indicate that this can not be the sole reason.

At 50°C and low ATP concentration the ratio of poly(A) synthesis with and without primer is at least 1000 and this is the highest ratio obtained in any system for RNA synthesis thusfar. We have no reason to doubt that this technique will also work with other templates, because at 50°C primer-independent synthesis with poly(dA) and a natural single-stranded DNA (not shown) were also strongly suppressed.

The protective effect of the oligoribonucleotide in our model system and that of Terao *et al.*[8] against the inhibitory action of rifampicin shows that this drug can be used as a second, although less effective, tool to restrict the transcription to the elongation of oligomer primers. With rifampicin present, the observed amount of *de novo* poly(A) synthesis did not exceed more than 6 or 9% of the primer-dependent synthesis at low or high ATP concentration, respectively.

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